# Rapid evolution of a floral trait following acquisition of novel pollinators

Article (Published Version)

Received: 1 September 2020 | Accepted: 9 February 2021

DOI: 10.1111/1365-2745.13636

RESEARCH ARTICLE

Journal of Ecology | BRITISH ECOLOGICAL SOCIETY

# Rapid evolution of a floral trait following acquisition of novel pollinators

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**Funding information**
H2020 Marie Skłodowska-Curie Actions, Grant/Award Number: 706365; Percy Sladen Memorial Fund; Botanical Society of Britain and Ireland

**Handling Editor:** Ignasi Bartomeus

## Abstract

1. Changes in the pollinator assemblage visiting a plant can have consequences for reproductive success and floral evolution. We studied a recent plant transcontinental range expansion to test whether the acquisition of new pollinator functional groups can lead to rapid adaptive evolution of flowers.
2. In *Digitalis purpurea*, we compared flower visitors, floral traits and natural selection between native European populations and those in two Neotropical regions, naturalised after independent introductions. Bumblebees are the main pollinators in native populations while both bumblebees and hummingbirds are important visitors in the new range. We confirmed that the birds are effective pollinators and deposit more pollen grains on stigmas than bumblebees.
3. We found convergent changes in the two new regions towards larger proximal corolla tubes, a floral trait that restricts access to nectar to visitors with long mouthparts. There was a strong positive linear selection for this trait in the introduced populations, particularly on the length of the proximal corolla tube, consistent with the addition of hummingbirds as pollinators.
4. *Synthesis*. The addition of new pollinators is likely to happen often as humans influence the ranges of plants and pollinators but it is also a common feature in the long-term evolution of the angiosperms. We show how novel selection followed by very rapid evolutionary change can be an important force behind the extraordinary diversity of flowers.

**KEYWORDS**
bumblebee, contemporary evolution, *Digitalis purpurea*, floral evolution, hummingbird, pollinator change

## 1 | INTRODUCTION

Changes in pollinator communities and plant range shifts are currently affecting pollination interactions across many ecosystems (González-Varo et al., 2013; Goulson et al., 2015; Grass et al., 2014) and, in consequence, many plants are experiencing new pollinator environments. From the plant's perspective, rapid changes in pollination interactions can have implications on crucial processes such as reproductive success and, eventually, their evolution. Even over very short time-scales, new pollinator environments can potentially

lead to novel selection pressures on mating strategies and floral traits. For instance, there is evidence of range changes favouring shifts to increased levels of uniparental reproduction if pollinators become scarce or are completely lost, both via increased cloning (Castro et al., 2016; Ferrero et al., 2020) or via shifts to self-pollination (Bodbyl Roels & Kelly, 2011; Petanidou et al., 2012; Ward et al., 2012). In the latter cases, the degree of self-compatibility or floral morphological traits that favour selfing, such as the distance between anthers and stigmas, shows adaptive changes that provide reproductive assurance in the absence of pollinators. We are less certain about the implications for floral evolution in situations where the pollinator community changes to include new functional groups of floral visitors, which could select for new floral traits without necessarily changing the breeding system.

Over long time-scales, pollinators are important agents of selection of floral morphological traits that increase the mechanical fit between flower and pollinator, regulate access to rewards and optimise the attractiveness of the floral display. Evidence for this comes both from macroevolutionary patterns of adaptation to different pollinator functional groups (e.g. pollination syndromes, reviewed by Fenster et al., 2004) and adaptive intraspecific geographical variation resulting from historical local dissimilarity between pollinators, producing floral ecotypes (e.g. Anderson et al., 2010; Herrera et al., 2006; Paudel et al., 2016; Valiente-Banuet et al., 2004). Furthermore, artificial selection experiments consistently show that floral morphological traits can evolve in response to a changed pollination environment in a few generations (e.g. Gervasi & Schiestl, 2017; Lehtilä & Holmén Bränn, 2007; Lendvai & Levin, 2003; Worley & Barrett, 2000). In principle then, changes in the pollinator environment can be expected to lead to very rapid evolution of floral traits in wild populations as well. Much of the research addressing this question in the field has focused on potential changes in the mating strategies and reproductive morphology that rapidly occurs when invasive plants lose animal pollination altogether and resort to self-pollination (Issaly et al., 2020). However, to our knowledge, no studies have investigated the short-term evolutionary consequences for plants of the addition of entirely new functional pollinator groups, as opposed to a reduction in pollinator diversity. This is relevant not only in the context of global pollinator changes but also because rapid adaptation to new pollinator environments could be a key driver of angiosperm floral diversity. Such adaptation is most likely to happen in response to selection for novel phenotypes as plants are exposed to new pollinators (Harder & Johnson, 2009) while not necessarily losing their previous ones.

A unique opportunity to address this question comes from recent plant range expansions into areas where they are exposed to novel pollinator taxa. In this study, we use the short-lived herb *Digitalis purpurea* as a focal species to test whether a change in pollinator assemblage after the recent colonisation of a new continent leads to adaptive changes in floral morphology. In its native range in Western and Northern Europe, *D. purpurea* is pollinated by a few species of bumblebees (Broadbent & Bourke, 2012; Grindeland et al., 2005), but in naturalised populations in the Americas, hummingbirds have also become frequent floral visitors. With the addition of this new functional group of pollinators, our hypothesis is that the new pollinator environment will impose a different selection regime on flowers and lead to changes in floral traits, as for example, longer corollas typical of hummingbird-pollinated flowers. The convergent floral syndrome associated with hummingbird pollination across angiosperm families suggests that selection imposed by birds in particular can be strong (Caruso et al., 2019; Pauw, 2019), especially when there is poor morphological matching with the flower to begin with (Nattero et al., 2010). Alternatively, naturalised populations of *D. purpurea* could accommodate new pollinators without any detectable divergence in floral traits.

To test this, we focus on the comparison of native populations (from Southern England) with naturalised populations in two non-native areas where hummingbirds are reported as visitors (Colombia in South America and Costa Rica in Central America). We identified pollinators and quantified their visitation rates and pollen transfer effectiveness. In the same populations, we measured floral morphology and nectar characteristics, and quantified natural selection on these traits. Convergent variation in floral traits associated to new pollinators in independently evolving naturalised populations can provide evidence for rapid adaptation to novel pollination environments.

## 2 | MATERIALS AND METHODS

### 2.1 | Study system and field sites

*Digitalis purpurea* L. (Plantaginaceae) is a facultatively biennial herb that depends on light gaps or disturbed sites for germination and establishment. Seeds persist in the seed bank and can form densely populated aggregations. *Digitalis purpurea* is semelparous, with most individuals flowering only once in their lifetimes. On the second summer after germination (although this is sometimes delayed for one or more years), rosettes produce large showy inflorescences with several dozen flowers that open sequentially from the bottom to the top of the inflorescence. The purple flowers are bell-shaped and protandrous, with anthers dehiscing shortly after anthesis, while the stigma becomes receptive (by unfolding its two lobes) up to 5 days later (Darwin, 1876). The plant is self-compatible, but insect visitation is required for full seed set (Nazir et al., 2008; see also Section 3). Bumblebees typically fly upwards when foraging on an inflorescence, so on *D. purpurea* they travel from older female phase flowers lower in the inflorescence to male phase flowers higher up in the inflorescence, potentially reducing the incidence of self-pollination (Best & Bierzychudek, 1982). The main pollinator in the native European range is the garden bumblebee, *Bombus hortorum*, found to be the predominant visitor to plants in the UK and Norway (Broadbent & Bourke, 2012; Grindeland et al., 2005; Manning, 1956). The same studies report that other *Bombus* species with long tongues, such as *B. pascuorum*, can also be frequent visitors and pollinators of *D. purpurea*. Visitation by insects with shorter


**FIGURE 1** Longitudinal section of *Digitalis purpurea* flower with part of the corolla and one stamen removed. The floral nectaries are located at the base of the ovary, within the constricted proximal part of the corolla tube

mouthparts is likely restricted by the narrow restriction of the corolla tube at its proximal part (Figure 1).

*Digitalis purpurea* is native to Western Europe, including the British Isles, but has become naturalised in many temperate regions and tropical highlands of the world (Bräuchler et al., 2004; Heywood, 1951). Populations in South and Central American mountains likely originate from garden escapees imported by English engineers (Calle et al., 1989; Díaz, 2011). Precise dates of the introductions are not available for either country, but no records of the plant are present in Ørsted's (1863) thorough description of the flora of Costa Rica from 1846 to 1848. Pérez-Arbeláez (1978) cites a botanical collection in Colombia from 1856 where the plant is described as a recent introduction. The first herbarium records date from 1928 in Costa Rica (www.tropicos.org) and 1932 in Colombia (Virtual Herbarium, Universidad Nacional de Colombia). It is thus likely that the introductions happened sometime around the 1850s. Because this is a biennial species, we can assume there have been <85 generations in the introduced areas. The two regions included in this study are separated by strong geographical barriers, including the vast lowland forests in the Panama isthmus where *D. purpurea* would not survive so that it is highly unlikely that the populations in Central and South America have a single origin with subsequent natural dispersal. Human-mediated dispersal from one region to the other would still be a possibility, but preliminary molecular results firmly points towards independent introductions. A dataset comprising ~9K single nucleotide polymorphisms (SNPs) confirms that UK populations are ancestral to both naturalised regions, and that populations in Colombia, Costa Rica and the UK cluster together within each region (=country), with very low or no admixture with the other two regions. The two tropical regions are also strongly divergent in this multilocus analysis, and given their recent establishment, this further supports the fact they originated from independent introduction events (M. C. Castellanos, unpubl.).

In the new range, plants can flower throughout the year and hummingbird visitation is frequently observed (Castellanos M.C., pers. obs.; Riveros et al., 2006). In Andean Colombia, the bumblebee species *Bombus hortulanus*, *B. atratus* and *B. rubicundus* have been reported to visit and rob *D. purpurea* flowers (Riveros et al., 2006).

In all, 11 populations of *D. purpurea* from the native and non-native range were chosen for comparisons of pollinator assemblage and floral morphology (Table 1). In a subset of them, we measured nectar and vegetative traits, and performed experiments to detect potential changes in the breeding system and pollen limitation. In four of these populations, we also measured natural selection on floral traits (Table 1). Fieldwork took place between 2016 and 2019.

## 2.2 | Breeding system

We used controlled hand pollinations to study the breeding system and assess the potential pollen limitation in three of the study populations (two native, one introduced; Table 1). Four different pollination treatments were applied to individual flowers on the same individual plants, for 8–20 individuals per population. The treatments were as follows: (a) an emasculated flower manually outcrossed using fresh pollen from another plant ('manually outcrossed'), (b) an emasculated flower manually selfed using pollen from another flower on the same plant ('manually selfed'), (c) a non-pollinated flower with normal anthers to allow for autonomous selfing ('naturally selfed') and (d) an open 'control' flower. Flowers in treatments (a) to (c) were covered with bridal veil bags while still in bud to prevent any pollinator visits. We removed bags after the flowers wilted to allow normal fruit development. Flowers in treatments (a) and (b) were emasculated by removing undehisced anthers using tweezers while still in bud, and hand-pollinated a few days later when the stigmas became receptive. After 4–8 weeks, we collected undehisced fruit capsules and left them to dry in separate paper envelopes. The seeds were extracted from fruits in the laboratory, photographed and counted using ImageJ 1.52e software (http://rsb.info.nih.gov/ij/). Seed counts were compared across treatments with linear models in R.

## 2.3 | Characterising pollinator assemblages and quantifying visitation

We quantified pollinator activity when the populations were in full bloom by surveying *D. purpurea* plants during a series of

**TABLE 1** *Digitalis purpurea* populations included in this study, with datasets collected. Coordinates are in WGS 84 degrees

| Region | Population | Coordinates (latitude, longitude) | Elevation (m) | Pollination censuses | Floral morphology | Nectar traits | Vegetative traits | Selection on traits | Breeding system |
|---|---|---|---|---|---|---|---|---|---|
| UK | Loder Valley | 51.055, −0.093 | 98 | ✓ | ✓ | ✓ | ✓ | ✓ | |
| (native) | Holy Cross | 50.972, 0.199 | 128 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | Calcot Wood | 50.921, −0.332 | 25 | ✓ | ✓ | | | | ✓ |
| | Ashdown Forest | 51.089, 0.153 | 113 | | ✓ | | | | |
| Colombia | Choachí | 4.592, −74.031 | 3,270 | ✓ | ✓ | ✓ | ✓ | ✓ | |
| (non-native) | Floresta Reserve | 4.802, −73.998 | 3,050 | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| | Guatavita | 4.979, −73.773 | 3,000 | | ✓ | | | | |
| | La Vieja | 4.711, −74.011 | 3,025 | | ✓ | | | | |
| | Encenillo | 4.789, −73.909 | 3,080 | | ✓ | | | | |
| Costa Rica | La Georgina | 9.559, −83.724 | 3,070 | ✓ | ✓ | ✓ | ✓ | ✓ | |
| (non-native) | Cuericí | 9.555, −83.667 | 2,565 | | ✓ | | ✓ | | |

3-min censuses (June and July in the UK, December and February in Colombia, March and April in Costa Rica) covering all times of the day that floral visitors are active, including dawn in the tropical populations. Surveys took place over 4–9 days during a single flowering season between 2016 and 2019 for most populations, except for Calcot Wood and Holy Cross in the UK that were studied on two consecutive summers. For each census, we surveyed multiple inflorescences containing 20–50 flowers and recorded (a) the number of flowers surveyed, (b) the species of visitor and (c) the number of flowers visited in the 3-min period. We then estimated legitimate visitation rates as the number of visits per flower per hour. These were compared between populations using generalised linear models followed by Tukey's pairwise comparisons using function *glht* from the R package MULTCOMP (Hothorn et al., 2008).

For visiting bumblebee species in the non-native range with no published functional morphological measurements, we collected specimens and measured their tongue lengths (glossa plus prementum) for comparisons with pollinators in the native range.

## 2.4 | Effectiveness of pollinators

As one measure of their pollination effectiveness, we compared the ability of common visitors at delivering pollen to virgin stigmas after a single visit in two native UK populations, and three non-native populations. For this, we emasculated flowers while still in the bud stage and bagged them to prevent any visits. Once the stigma on a flower had become receptive a few days later, bags were removed and the plant monitored for visits from a pollinator. Immediately after a single legitimate visit, we identified the pollinator and squashed the flower's stigma on a microscope slide using fuchsin-stained glycerine jelly. This was repeated for as many pollinator species as possible, and all conspecific pollen grains were counted under a microscope with help from photographs if needed. We tested for differences among functional groups of pollinators using analysis of variance and running paired Tukey tests in the BASE package in R.

## 2.5 | Comparisons of floral morphology and nectar traits

A minimum of 40 healthy plants were chosen haphazardly from each of the 11 populations (between 2015 and 2019) for morphological characterisation. Between three and four flowers were collected from different positions in each inflorescence to account for any intra-plant variation in floral traits. Picked flowers were pressed in filter paper, dried in an oven at 45°C for at least 2 days, and for a further 1 day immediately before measuring. After drying, flowers were weighed on a precision balance to the nearest 0.001 g for a measure of dry weight.

We then used digital images of the pressed flowers to measure whole corolla length, whole corolla height, proximal corolla tube length and proximal corolla tube width, using ImageJ software (Figure 2). Strictly speaking, we are measuring the *height* of the proximal corolla tube (see Figure 2), but because this section of the corolla tube is roughly cylindrical, the width and the height are approximately the same. We refer to it as *width* for consistency with previous studies in corolla evolution. As expected, all four traits covary significantly to some extent within each population, with the strongest correlations occurring between whole corolla height and whole corolla length (up to $r = 0.71$), and proximal corolla length and proximal corolla width (up to $r = 0.51$). For the morphological comparison analysis, we therefore use the geometric mean of the length and height of the whole corolla ('whole corolla size' hereafter) and the geometric mean of the length and width of the proximal corolla tube ('proximal corolla tube size' hereafter). We keep the whole corolla tube size and proximal corolla tube size as separate traits, because the proximal tube is the constricted part at the base of the corolla tube restricting access to the nectaries for floral visitors with

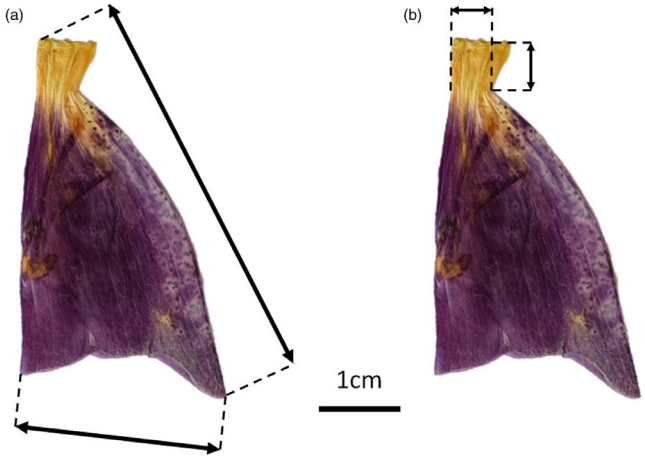


**FIGURE 2** Pressed *Digitalis purpurea* flowers illustrating the morphological measurements taken. (a) Whole corolla length and height; (b) Proximal corolla tube length and width

short mouthparts (see Figure 1), and thus we are interested in the functional role it may play in different pollinator environments. Two vegetative traits were also measured for all plants in each population: rosette diameter (taken as the longest linear measurement from leaf tip to leaf tip across the rosette), and inflorescence height to the first flower (i.e. the peduncle, equivalent to the height from the base of rosette to the first flower on the inflorescence).

Trait measures were compared between the native and non-native range using mixed-effects linear models with population and individual plant as random factors. Linear models were programmed using the 'lmer' function in the LME4 R package (Bates et al., 2015).

We measured nectar traits in five of the study populations (Table 1). We bagged inflorescences and after 24 hr, we picked two to three flowers in the male phase from each plant and used microcapillary tubes to measure the volume of nectar from the base of the corolla. We then used a pocket refractometer to estimate the sugar concentration.

### 2.6 | Natural selection on floral traits

We estimated total seed production by all focal plants in five of the study populations (Table 1) as a proxy for lifetime female fitness. Three ripe but undehisced fruits were collected from each of three different positions in the inflorescence (lower, middle and upper). This was done to account for any intra-plant variation in resources allocated to fruits at different ages of the plant, as our previous observations suggested that fewer seeds may be produced by fruits later in the season. To obtain total seed production, we multiplied the average number of seeds produced by these three fruits by the number of successful fruits produced in the lifetime of an individual plant. We estimated the total number of fruits from the numbers of flowers, as the initial count of flowers and fruits correlates strongly with the number of fruits produced ($r^2 = 0.95$, $p < 0.001$, $N = 116$, in two UK populations).

We measured both linear and nonlinear natural selection acting on floral traits in each population using lifetime seed production as a measure of female fitness. We estimated selection parameters using the general additive model (GAM) approach on absolute fitness values implemented by Morrissey and Sakrejda (2013). We report linear ($\beta$) and quadratic ($\gamma$) selection gradients on corolla traits estimated in bivariate models that included both whole corolla size and proximal corolla size, to control for correlations between the traits. Selection gradients estimate selection on each of the two traits, considering the other one simultaneously, and therefore estimate direct selection on each (Lande & Arnold, 1983). In addition, we ran separate univariate selection analyses for the four corolla tube traits in each population, to get further insight into the targets of selection. For nectar traits, we also estimated selection gradients in bivariate models where both volume and concentration were included. We fitted GAM models using the MGCV package in R (Wood, 2011), and then calculated the statistical significance of selection coefficients via the bootstrap approach ($N > 500$ iterations) implemented in package GSG (Morrissey & Sakrejda, 2014). Standardisation of traits and fitness values, as well as scaling of quadratic coefficients (as explained in Stinchcombe et al., 2008), are automatically implemented by the 'gsg' calculations.

## 3 | RESULTS

### 3.1 | Breeding system

Results of hand pollinations in two native *D. purpurea* populations (Holy Cross and Calcot Wood) were compared with one population in the introduced range (Floresta). Across all populations, hand pollination treatments produced significantly different numbers of seeds (estimate = 801.5; $p < 0.001$; $N = 8$–20 flowers per treatment in each population; Figure 3). Post-hoc tests show that flowers bagged for autonomous selfing ('naturally selfed') produced significantly fewer seeds than the control both in all populations in the UK ($p < 0.001$) and Colombia ($p < 0.001$), often aborting and not producing any viable seeds. This is consistent with previous reports for this species in the native range (Darwin, 1876) and confirms that non-native populations are also dependent on pollinators for seed production.

The manually self-pollinated flowers produced similar numbers of seeds (909 ± 90 in the UK and 559 ± 103 in Colombia) to the control (849 ± 90 for the UK and 736 ± 102 in Colombia) and outcrossed treatments (787 ± 63 in the UK and 801 ± 75 in Colombia), and differences between these were not statistically significant in post-hoc tests (all with $p < 0.001$); this confirms full self-compatibility (Darwin, 1876; Figure 3). The number of seeds produced in the manually outcrossed treatment and the control treatment was not significantly different in Colombia ($p = 0.93$) or the UK ($p = 0.92$), indicating no pollen limitation for this species in either the native or non-native range (Figure 3).

Figure 3 further shows that seed production was variable within treatments in both ranges and post-hoc tests confirmed that mean seed production in each treatment was not significantly different

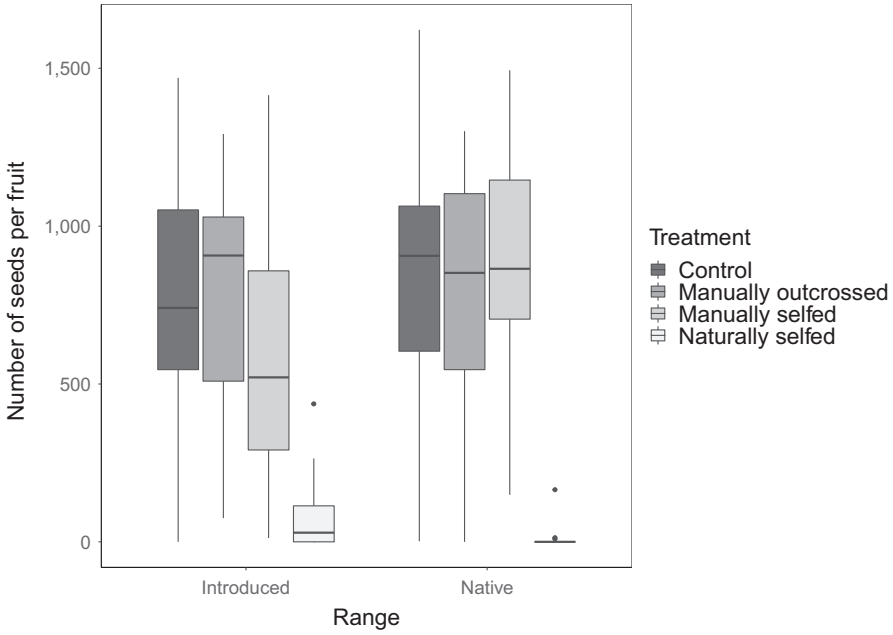


**FIGURE 3** Average seed production after four hand pollination treatments applied to *Digitalis purpurea* flowers in the new range (Floresta, Colombia) and the native range (averaged for two UK populations; $N = 8–20$ plants per population). See details of treatments in the text

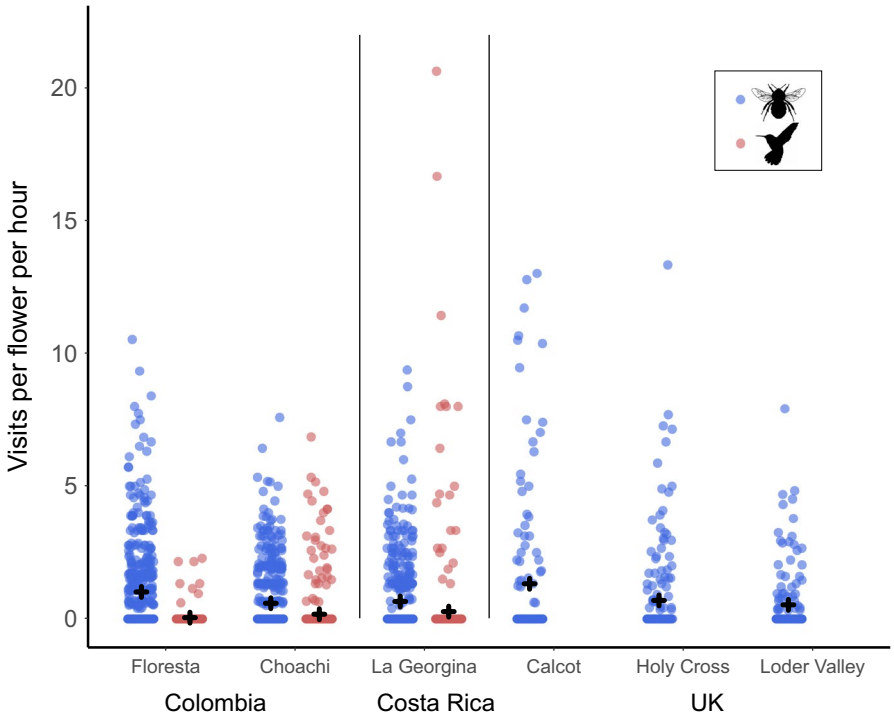


**FIGURE 4** Visitation rates (number of visits/flower$^{-1}$ × hour$^{1}$) by bumblebees and hummingbirds on *Digitalis purpurea* in each study population. Circles represent different pollinator censuses and are plotted with a horizontal jitter to help visualising overlapping values. Black crosses show the mean values for each population; medians are very close to zero in all cases due to many censuses recording no visits

across regions. Note that the spontaneous selfing treatment ('naturally selfed') showed more variance and higher mean values of seed set in the introduced population; however, this was not significantly different from the native populations.

## 3.2 | Pollinator assemblages and visitation

We quantified floral visitation from 3-min censuses that added up to 25–31 hr of observation per population in the non-native range (Floresta: 524 censuses, Choachí: 624, La Georgina: 506), and 7–10 hr in native populations (Calcot Wood: 140, Holy Cross: 201, Loder Valley: 161). We ran more censuses in the non-native populations where visitor diversity was higher; plateaus in species accumulation curves show that with this sampling effort we were successful at recording an accurate representation of floral visitors in both native and tropical non-native populations (Supporting Information Figure S1).

Populations in tropical mountains have a more diverse group of floral visitors with up to seven species of legitimate pollinators, compared to two in the native range (Supporting Information Figure S1). Overall, bumblebees were the most frequent functional group of pollinators in all populations (Figure 4), with a single *Bombus* species

often dominating (Supporting Information Table S1). In non-native populations, hummingbirds performed up to 27% of the legitimate visits. Smaller bees and other insects were infrequent visitors in all populations; when they do visit, they often have trouble accessing the flowers due to the long hairs at the base of the corolla that act as barriers, or are too small to touch the stigmas and perform pollination.

Populations in the introduced range received significantly more pollinator visits on average (0.86 ± 1.66 *SD* visits per flower per hour; $N = 1654$) than populations in the native range (0.79 ± 1.99 *SD*; $N = 502$; $p = 0.005$; Figure 4). Flowers in the native range received significantly more visits by bumblebees on average than those in the introduced range ($p = 0.003$), with populations in the UK receiving a mean of 0.79 ± 1.99 *SD* visits per flower per hour and populations in the non-native range receiving 0.72 ± 1.38 *SD* bumblebee visits per flower per hour (Figure 4). There was variation in visitation rates across populations within regions as well (Figure 4).

Measurements of functional morphological traits for *Bombus robustus* showed that they have an average tongue length of 6.9 mm ± 0.55 *SD* ($N = 6$). All other bumblebee species recorded in both ranges already have morphological measurements published in the literature (see summary in Supporting Information Table S2). The tongue length of bee species in the introduced range has means of 6.9–11.1 mm compared with 7.89–12.9 mm for those the native range (Table S2).

Nectar robbing by making holes at the base of the corolla was frequent in some of the non-native populations (e.g. 10.4% of all visits to flowers in Floresta), whereas it was absent in the native range (Supporting Information Table S1). Casual observations in Floresta found that 64% of plants had at least one flower robbed ($N = 50$), and 12% of plants in the sample had 100% of open flowers robbed. Some visitors acted both as pollinators and robbers. In some cases, pollinators switched from visiting flowers legitimately to robbing in the same foraging bout, and in others they performed a single foraging behaviour.

## 3.3 | Effectiveness of pollinators

Hummingbird species of *Eriocnemis* and *Aglaeactis* in Choachí, Colombia, deposited a significantly larger number of pollen grains on average in single visits (4,380 ± 2,964 *SD*, $N = 17$) than bumblebees in native (728 ± 1,053 *SD*, $N = 38$, adjusted $p < 0.001$) or non-native populations in Colombia (1,780 ± 3,179 *SD*, $N = 95$, adjusted $p < 0.001$; Figure 5). Pollen deposition was variable within each functional group, but bumblebee data showed particularly large variation in the Colombian populations, from several hundreds to >13,000 grains deposited in a single visit. These data come from at least four different species of bumblebee (*Bombus funebris*, *B. hortulanus*, *B. rubicundus* and *B. robustus*) and could reflect local variation in flower abundance and also depend on the seasonal presence of large-bodied queens.

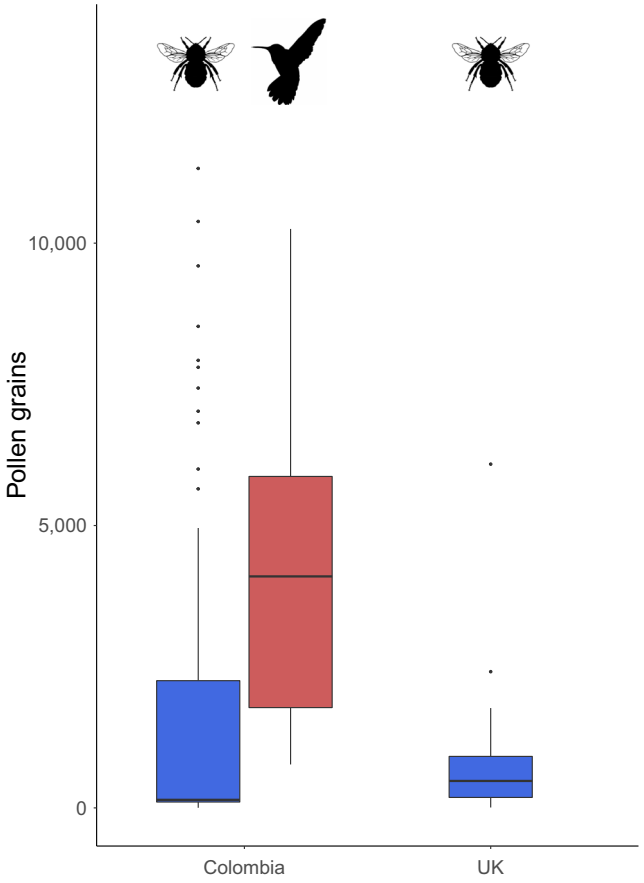


**FIGURE 5** Pollen deposition on *Digitalis purpurea* stigmas after single visits by hummingbirds (red) in Colombia ($N = 17$) and bumblebees (blue) in Colombia ($N = 95$) and the UK ($N = 38$). Bumblebee icon modified from www.divulgare.net

## 3.4 | Comparisons of morphology and nectar traits

Whole corolla tube size did not differ between the introduced ($N = 783$ flowers in 250 plants across seven populations) and the native range ($N = 559$ flowers in 165 plants in four populations, estimate = 1.24; $p = 0.49$, Figure 6a). By contrast, the proximal corolla tube was 13% and 26% larger on average (in Colombia and Costa Rica, respectively) in the introduced range ($N = 649$ flowers in 201 plants and 146 flowers in 49 plants, respectively) as compared with the native range ($N = 579$ flowers in 166 plants; estimate = 0.84; $p = 0.004$; Figure 6b). We found no significant differences between the native and introduced range for the volume of nectar ($N = 31$ and 142 plants, respectively; estimate = 1.64, $p = 0.22$) or its concentration (estimate = 5.1, $p = 0.31$). Similarly, there were no significant differences between ranges in whole-plant vegetative traits: inflorescence height to the first flower (peduncle, $N = 79$ and 150 plants, respectively; estimate = 7.92, $p = 0.47$) and rosette diameter (estimate = 7.46, $p = 0.06$; Supporting Information Figure S2).

Overall, bigger plants (i.e. those with larger vegetative traits) did not consistently have larger corollas, across or within populations: whole corolla tube was significantly correlated with inflorescence

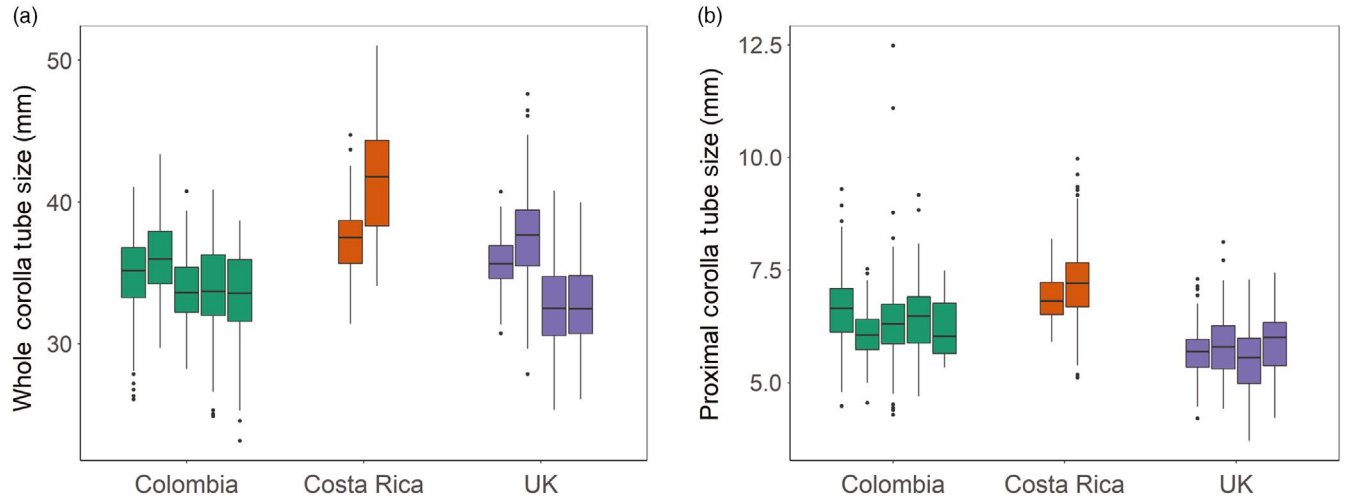


**FIGURE 6** Comparison of (a) whole corolla tube size and (b) proximal corolla tube for all *Digitalis purpurea* populations (each in a separate boxplot) in the three regions

**TABLE 2** Directional and quadratic selection gradients or coefficients (±*SE*) for the floral traits in each population of *Digitalis purpurea*. Values that are statistically significant from zero are in bold type with *p* values indicated by *($p < 0.05$), **($p < 0.01$) and ***($p < 0.001$)

| Trait | Country | Population | Directional ($\beta$) | NonLinear ($\gamma$) |
|---|---|---|---|---|
| Whole corolla tube size | UK | Loder Valley | −0.21 ± 0.124 | 0.00 ± 0.000 |
| | UK | Holy Cross | 0.00 ± 0.111 | 0.00 ± 0.000 |
| | Costa Rica | La Georgina | 0.05 ± 0.125 | 0.00 ± 0.000 |
| | Colombia | Floresta | −0.03 ± 0.064 | 0.00 ± 0.000 |
| | Colombia | Choachí | −0.07 ± 0.049 | −0.06 ± 0.046 |
| Proximal corolla tube size | UK | Loder Valley | 0.21 ± 0.119 | 0.00 ± 0.001 |
| | UK | Holy Cross | −0.05 ± 0.106 | 0.00 ± 0.000 |
| | Costa Rica | La Georgina | **0.22 ± 0.131*** | 0.00 ± 0.000 |
| | Colombia | Floresta | **0.32 ± 0.080***** | −0.02 ± 0.046 |
| | Colombia | Choachí | **0.14 ± 0.043**** | 0.00 ± 0.000 |
| Proximal corolla tube length | UK | Loder Valley | 0.15 ± 0.123 | 0.00 ± 0.000 |
| | UK | Holy Cross | −0.02 ± 0.096 | 0.00 ± 0.000 |
| | Costa Rica | La Georgina | **0.21 ± 0.13*** | 0.00 ± 0.000 |
| | Colombia | Floresta | **0.27 ± 0.058***** | −0.01 ± 0.008 |
| | Colombia | Choachí | **0.10 ± 0.048*** | 0.00 ± 0.000 |
| Proximal corolla tube width | UK | Loder Valley | −0.03 ± 0.118 | 0.00 ± 0.000 |
| | UK | Holy Cross | −0.07 ± 0.102 | 0.00 ± 0.000 |
| | Costa Rica | La Georgina | 0.20 ± 0.127 | 0.00 ± 0.000 |
| | Colombia | Floresta | **0.14 ± 0.067*** | 0.00 ± 0.000 |
| | Colombia | Choachí | 0.06 ± 0.045 | −0.03 ± 0.022 |
| Nectar volume | Costa Rica | La Georgina | −0.10 ± 0.128 | 0.00 ± 0.000 |
| | Colombia | Floresta | −0.05 ± 0.075 | 0.00 ± 0.000 |
| | Colombia | Choachí | **0.16 ± 0.046***** | 0.01 ± 0.012 |
| Nectar concentration | Costa Rica | La Georgina | 0.06 ± 0.135 | −0.09 ± 0.072 |
| | Colombia | Floresta | 0.06 ± 0.070 | 0.00 ± 0.000 |
| | Colombia | Choachí | 0.05 ± 0.044 | 0.01 ± 0.006 |

height to the first flower in the complete dataset ($r = 0.19$, $p < 0.01$), but no other pair of vegetative and floral trait was significantly correlated. Interestingly, we found that plants in non-native populations produced, on average, 58.2% fewer flowers and fruits than plants in native populations (across population means: UK = 124.1 fruits per plant with $N = 76$; Colombia = 53.0 flowers per plant with $N = 95$,

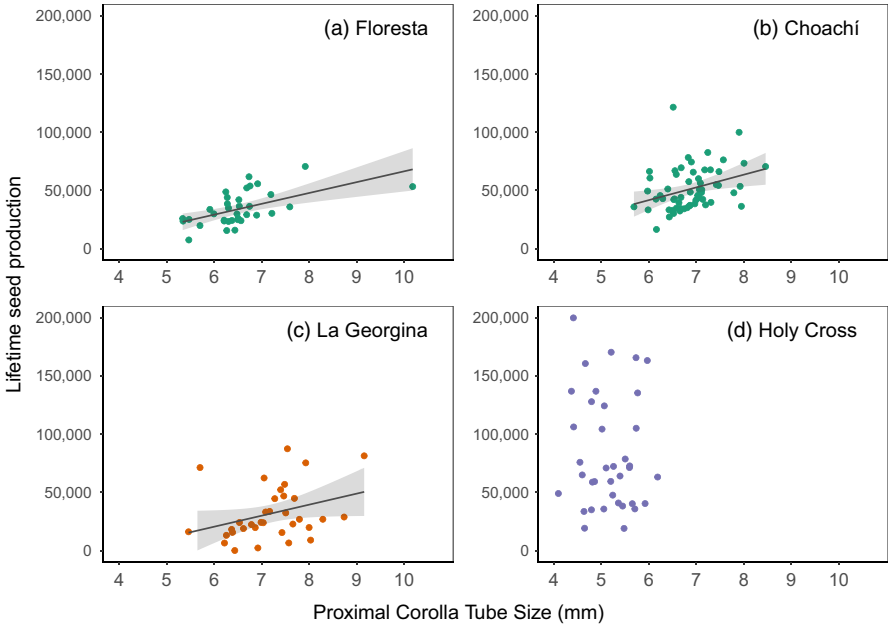


**FIGURE 7** Lifetime seed production (numbers of seeds) against proximal corolla tube size, measured as the geometric mean of proximal corolla tube length and width. Non-native populations of Digitalis purpurea (a) Floresta, Colombia, (b) Choachí, Colombia, (c) La Georgina, Costa Rica; and native (d) Holy Cross in the UK. The lines represent significant linear components of selection models, with the non-significant quadratic term excluded

Costa Rica = 48.8 with $N = 34$; $p < 0.001$) while producing the same mean number of seeds per fruit ($p > 0.5$). As a consequence, plants in the introduced range produce 64.2% fewer total seeds ($p < 0.001$). This is in spite of plants not being consistently smaller in the new range.

### 3.5 | Natural selection on floral traits

We found no linear or quadratic selection on the whole corolla size in any population in the native or non-native range (Table 2; Supporting Information Figure S3). We found significant positive linear selection gradients on the size of the proximal corolla tube for all three non-native populations studied (Figure 7; Table 2): in Floresta with a selection gradient of 0.32 ($p < 0.001$), in Choachí with a selection gradient of 0.14 ($p = 0.008$) and in La Georgina with a selection gradient of 0.22 ($p = 0.04$). In contrast, there was no evidence of selection on proximal corolla tube size in the UK populations (Figure 7; Table 2; Supporting Information Figure S4), indicating that the proximal corolla tube is not under selection in the native region. Nonlinear analysis showed no evidence for stabilising or disruptive selection on the proximal corolla size in any population (Table 2).

The selection analysis in the previous paragraph used the geometric means of corolla traits, as explained in the Methods. To get further insight on which aspect of the corolla is the target of selection, we ran separate univariate selection models for the height and the length of the whole corolla and the length and width of the proximal corolla tube in each population. We found that selection is concentrated mostly on the length of the proximal corolla; it was significant in all three non-native populations (Table 2; Floresta: selection coefficient 0.27, $p < 0.001$; Choachí: selection coefficient of 0.10, $p = 0.03$; La Georgina: selection coefficient 0.21, $p = 0.04$), and significant on the width of the proximal corolla in only one of them (Floresta: selection coefficient 0.14, $p = 0.02$). For the whole corolla, univariate models found significant selection only on the height in one non-native population (Choachí: selection coefficient of −0.10, $p = 0.03$; Supporting Information Table S3).

We found variable evidence of selection on nectar traits in the non-native populations (Table 2). Nectar volume was under strong positive linear selection in one Colombian population (Choachí: selection gradient 0.16, $p < 0.001$), but not in the other populations. Nectar concentration was not under linear or nonlinear selection in any non-native population either. Native populations were not included in this analysis, as we did not measure nectar traits in the same individual plants where we estimated seed production.

## 4 | DISCUSSION

We found evidence of evolution in floral morphology when comparing *D. purpurea* populations in the native range with two recently colonised regions. Plants in the new range, visited by a pollinator assemblage that includes a new functional group—hummingbirds—and novel bumblebee species, had proximal corolla tubes that are 13%–26% larger than plants in the native range, where they are pollinated solely by bumblebees. This corolla trait plays a role in determining access to nectar, as only pollinators with long enough tongues or beaks can reach the nectaries at the base of the ovaries. Consistent with this morphological change, we also found directional selection acting to increase the proximal corolla length in the three populations we tested in the new range, whereas corolla traits in the native range are not currently under detectable selection. Our study is the first to investigate contemporary evolution of flowers after the addition of a new functional pollinator group, and we find changes that are consistent with rapid evolutionary change in response to the new pollinator environment.

Evidence of population differences in corolla morphology consistent with variation in pollinator morphology is abundant in the literature; examples include, among many others, (a) *Gladiolus longicollis*

corolla tube ecotypes that are maintained by differences in the local moth community (Anderson et al., 2010), (b) *Nicotiana glauca* populations with dissimilar corolla lengths when visited by hummingbirds with correspondingly different bill lengths (Nattero et al., 2011) and (c) *Narcissus papyraceus* populations with larger tepal and corolla tubes when visited by moths rather than syrphid pollinators (Pérez-Barrales et al., 2007). Our *D. purpurea* data show that these adaptive changes can take place rapidly, in the short time since colonisation in the introduced range (<85 generations). In this case, larger proximal corolla tubes might be favoured by an improved efficiency of pollen transfer by novel pollinators. The bumblebee species in the new range have similar functional morphological traits to the bees in the native range, as their tongue lengths and body sizes do not differ significantly (del Castillo & Fairbairn, 2012; see also Table S2). However, hummingbirds constitute an important functional group in the new range, where they perform on average 22% of the visits and are more effective than bumblebees at depositing pollen on stigmas. Hummingbirds have been shown in the past to be more effective at delivering pollen to other flowers compared to bumblebees, even if they remove the same amount, making them overall more effective pollinators (Castellanos et al., 2003). The birds could thus be exerting selective pressures for easier access to *D. purpurea* nectar and better morphological fit while hovering, that is, plants that have longer and less constricted proximal corolla tubes. Here we do not yet provide direct evidence of this mechanism for selection by the hummingbirds, but will be testing it with selective exclusion of pollinators in the future. Interestingly, we found no differences or selection acting on the whole corolla tube, suggesting that fit and access to nectar rewards is determined mainly by the proximal base of the corolla in this species. Selection on this proximal part of the corolla is consistent across distant populations, even though there is no pollen limitation for seed quantity in any of them. One possible reason for selection to be occurring in the absence of pollen limitation is that hummingbirds could also be enhancing seed quality by reducing geitonogamy if pollen is moved farther from the parental plants, as seen in multiple bird-pollinated plants (reviewed by Krauss et al., 2017; Pauw, 2019). This aspect and a potential effect on the male components of reproductive success are yet to be tested in this species.

These findings are consistent with patterns of selection typically imposed by hummingbirds and other bird pollinators, who have favoured the evolution of flowers with long corollas across multiple angiosperm lineages, in one of the best examples of floral evolutionary convergence (Fenster et al., 2004; Grant & Grant, 1968). We found no differences between native and introduced populations in other floral traits that are often associated to hummingbird pollination, such as nectar volume or quality. Overall, *D. purpurea* flowers produce large enough volumes of nectar to be attractive to hummingbirds (3.8–7.4 μl in 24 hr without visitation, pers. obs.). There was a significant directional selection for higher nectar volume in one non-native population, but this was not consistent in all studied populations. Because the hummingbird communities visiting *D. purpurea* are different in each population, this warrants further investigation in the future. However, nectar traits are highly sensitive to environmental conditions (e.g. water availability, temperature, etc.; reviewed in Parachnowitsch et al., 2019) and are thus likely to require long-term consistent selection for a detectable response. This is in contrast to linear morphological traits that often present high values of heritability, even when measured in field conditions (Ashman & Majetic, 2006; Castellanos et al., 2019), and that have been shown to change in response to single mutations with implications for pollinator visitation (see Ding et al., 2017 for an example in *Mimulus*). For *D. purpurea* we are in the process of measuring heritability both in the field (using molecular markers) and in a common garden, and preliminary results from the field studies point towards very high and significant narrow sense heritabilities ($h^2 > 0.45$) for linear corolla traits (Castellanos M.C., unpubl.). This is consistent with the rapid evolution observed in the newly colonised populations.

The capacity for rapid evolution in corolla traits has been corroborated by several studies which imposed artificial selection under greenhouse conditions and found a quick response, including changes to corolla diameter, area (Lehtilä & Holmén Bränn, 2007; Lendvai & Levin, 2003; Worley & Barrett, 2000) and length (Conner et al., 2011). Evidence of very rapid evolution in natural conditions, (faster than ecotype formation in species with large home ranges), however, has been mostly limited to other reproductive traits such as flowering phenology (Colautti & Barrett, 2013; Lustenhouwer et al., 2018), as well as self-compatibility to provide reproductive assurance for plants losing pollinators when invading a new area (Barrett et al., 2008; note that in self-compatible *D. purpurea* there is no change in the breeding system in the newly colonised area, where dependence on pollinators remains high). Examples of rapid evolution of corolla traits in the wild are scarce, with a remarkable exception provided by Campbell et al., (2018). Studying an *Ipomopsis* hybrid zone, they demonstrated contemporary evolution of corolla length in approximately five generations. Notably, these changes closely followed their predictions of evolutionary change based on previous measures of the strength of selection by pollinators and the high heritability of corolla length. Flowers in this species are under divergent selection along an altitudinal cline, where pollinator communities vary along the cline and impose varying selection regimes.

Although studies showing rapid evolutionary change in corolla traits in the wild are rare, several others have demonstrated short-term changes in selection after disruption of previous pollination environments. Temeles and Bishop (2019) measured natural selection on corolla length in *Heliconia wagneriana* in Dominica before and after Hurricane Maria, which changed the composition of the plant community and as an indirect consequence, the prevalent hummingbird visitor of *H. wagneriana* from 1 year to the next. Before the hurricane, plants experienced no selection on corollas, but after a short-billed hummingbird became the predominant pollinator, the morphological mismatch led to strong selection for shorter corollas. Similarly, a study by Murúa et al., (2010) showed how the predominant pollinators of a wild violet differ between human-transformed forests and native forests less than 4 km away. The change in pollinators has led

to relaxed selection on flower number and novel disruptive selection on corolla shape in the disturbed sites. If sustained over a long time period, the change in selective pressures documented in these two study cases could potentially lead to floral evolution at a local scale.

In both previous examples, it is certain that the evolving populations are the same or part of the original ones. However, in cases of long-distance colonisation there is potential for founder effects arising from the invasion process if, for example, only a subsample of the original phenotypes were introduced to the new range. Changes in corolla length of invasive *Nicotiana glauca* populations studied by Schueller (2007), for instance, could not be explained by changes in the pollinators alone and were instead consistent with an initial bottleneck. In the case of our *D. purpurea* study populations, three sources of evidence suggest that the observed changes in the proximal corolla tube are not simply the consequence of a stochastic founder event. First, the comparison of a set of ~9K SNP markers shows that populations in Colombia and Costa Rica are genetically distinct from each other and each closer to those in the UK (see Section 2; Castellanos, in prep.). This is consistent with separate introductions to the two tropical regions and the convergent change in proximal corollas. Second, we found no significant differences in vegetative traits between the native and introduced range. If present, differences in a number of traits could have suggested founder effects with phenotypically distinct plants colonising the new areas, or divergent evolution after invasion, but none seem to be the case in these tropical regions. Willis et al., (2000) also studied vegetative traits in non-native populations of *D. purpurea* in Australia and New Zealand, and found that growth traits showed no differentiation from UK and French populations after a post-invasion period similar to the one in this study. Vegetative traits in *D. purpurea* thus appear to vary little even when plants successfully colonise different continents. Finally, no other floral trait that we studied (whole corolla tube and nectar traits) showed differentiation between native and non-native populations, nor experienced significant selection across the non-native populations. A parallel change in whole corolla tube could be expected because it is correlated to the proximal part of the corolla to some extent and corolla traits tend to be highly integrated (Berg, 1960). Strong selection could be decoupling the evolution of the two parts of the corolla; however, testing this hypothesis will require further experimental work in the non-native populations.

### 4.1 | Concluding remarks

Our study adds to many previous studies that use range changes as an opportunity to study trait evolution in plants. Our findings also contribute to the growing evidence that plants invading new areas can rapidly evolve even after only decades since their establishment (Colautti & Lau, 2015), potentially favoured by genetic isolation from the original populations, and in spite of potential constraints such as genetic correlations among traits (Ashman & Majetic, 2006). Here we demonstrate that range changes can also be used to study reproductive resilience and floral evolution when new pollinators are acquired. The addition of new functional groups to a plant´s pollinator environment is likely to happen more often as plants or pollinators migrate due to human influence but it is also presumably a common feature in the long-term evolution of the angiosperms (Grant & Grant, 1965; Stebbins, 1970). During episodes of contact with new pollinators, even in the presence of previous ones, novel and creative selection can change the tempo of flower evolution (reviewed by Harder & Johnson, 2009). By focusing on a period of potential floral innovation, our study on *D. purpurea* shows that adaptation of key floral traits to new pollinators can happen rapidly in response to sustained selection. Further studies on contemporary evolution in plants acquiring novel pollinators can add more evidence to confirm that selection for novel phenotypes followed by rapid evolutionary change can be an important force behind the extraordinary diversity of flower form and function.


## ACKNOWLEDGEMENTS

We are grateful to Loder Valley Nature Reserve (Kew-Wakehurst), Holy Cross Priory Care Home, Wiston Estate, AsoFloresta, Estación Biológica Encenillo, Restaurante La Georgina, and Carlos and Ana Solano from Estación Biológica Cuericí for access to their land and *D. purpurea* populations. We thank Pablo Stevenson (Universidad de Los Andes) and Martha Giraldo for ongoing support with fieldwork in Colombia. Esteban Saad, María José Mata, Karen Gil, Stephanie Núñez and Cynthia Silva provided valuable assistance during fieldwork in Colombia and Costa Rica. Fieldwork and collections were authorised by ANLA (Colombia) and SINAC and CONAGEBIO (Costa Rica). We thank Jeff Ollerton for useful comments that improved the manuscript. Financial support came from the Botanical Society of Britain and Ireland (BSBI), a travel grant from the Percy Sladen Memorial Fund, and the European Union's Horizon 2020 programme under the Marie Sklodowska-Curie grant agreement No 706365 to M.C.C.


## AUTHORS' CONTRIBUTIONS

C.R.M. and M.C.C. performed the field data collection with help from J.F.P., N.J.B. and M.A.B.; C.R.M. processed the samples; M.C.C. conceived the research; C.R.M. and M.C.C. performed the statistical analyses and wrote the manuscript. All authors critically commented on the manuscript and gave final approval for publication.

## PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/1365-2745.13636.

## DATA AVAILABILITY STATEMENT

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.zw3r2287h (Mackin et al., 2021).


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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Mackin CR, Peña JF, Blanco MA, Balfour NJ, Castellanos MC. Rapid evolution of a floral trait following acquisition of novel pollinators. *J Ecol*. 2021;00:1–13. https://doi.org/10.1111/1365-2745.13636